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(54) **Improvements in or relating to the growth of plant tissue cultures**

(57) A process for the production of a composite substrate containing immobilised plant cells, which process comprises contacting a glass fibre tissue (as hereinbefore defined) with an aqueous solution of sodium or potassium alginate containing suspended plant or algal cells, and forming an alginate gel on the tissue by contacting the tissue with an aqueous solution of calcium chloride or other suitable non-phytotoxic salt.

The glass fibres may be made from an "E" glass composition. The fibre may be formed as a tissue by taking chopped strands in water and treating them with a melamine or styrene binder or is formed by a continuous filament drawing process from a composition containing a transition metal oxide.

The claims were filed later than the filing date within the period prescribed by Rule 25(1) of the Patents Rules 1982.

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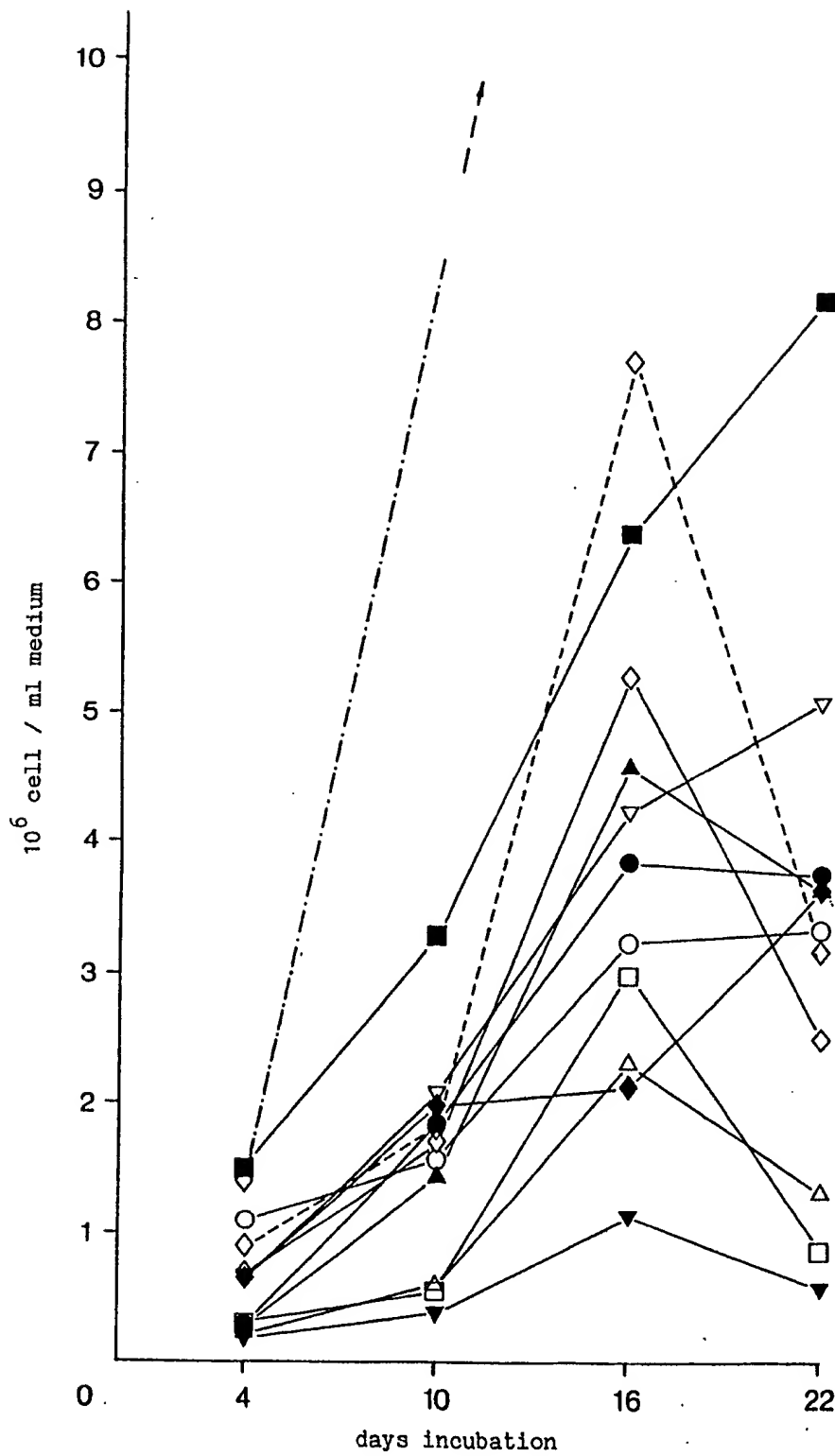


FIG. 1a

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KEY TO GRAPH OF FIG. 1a.

- E Glass, No Binder
- Titania Glass, No Binder
- ▲ E Glass, Styrene Binder
- △ Zirconia Glass, Polystyrene Binder
- ▼ E Glass, Melamine Binder
- ▽ C Glass, Acrylic Binder
- Titania Glass, Polystyrene Binder
- E Glass, Burnt Off
- ◆ E Glass, Burnt Off and Titanium coated
- ◇—◇ Control 1 (J-cloth)
- ◇- - -◇ Control 2 (Whatman No. 1)
- ◇- · -◇ Control 3 (Alginate only)

FIG. 1b

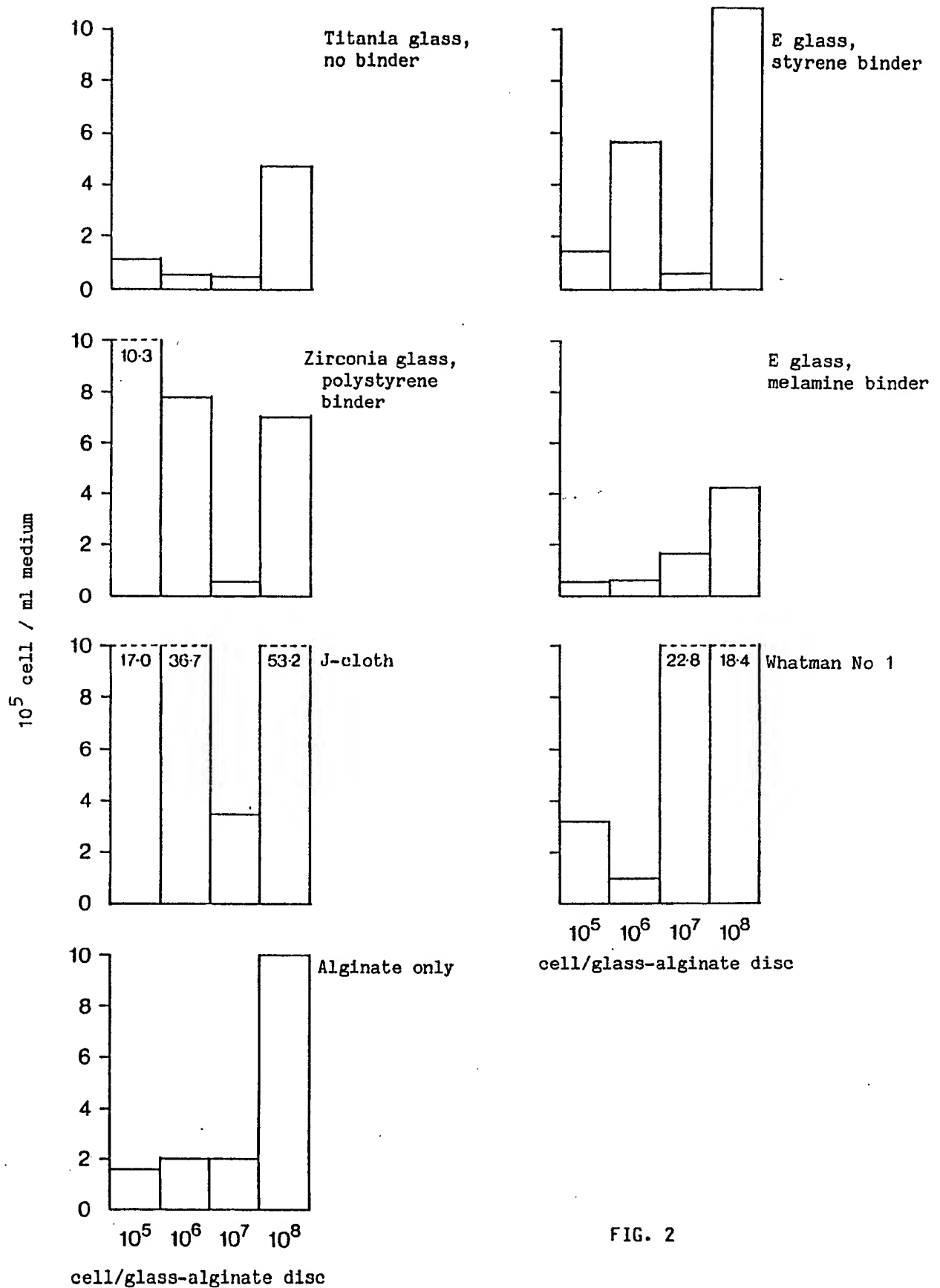


FIG. 2

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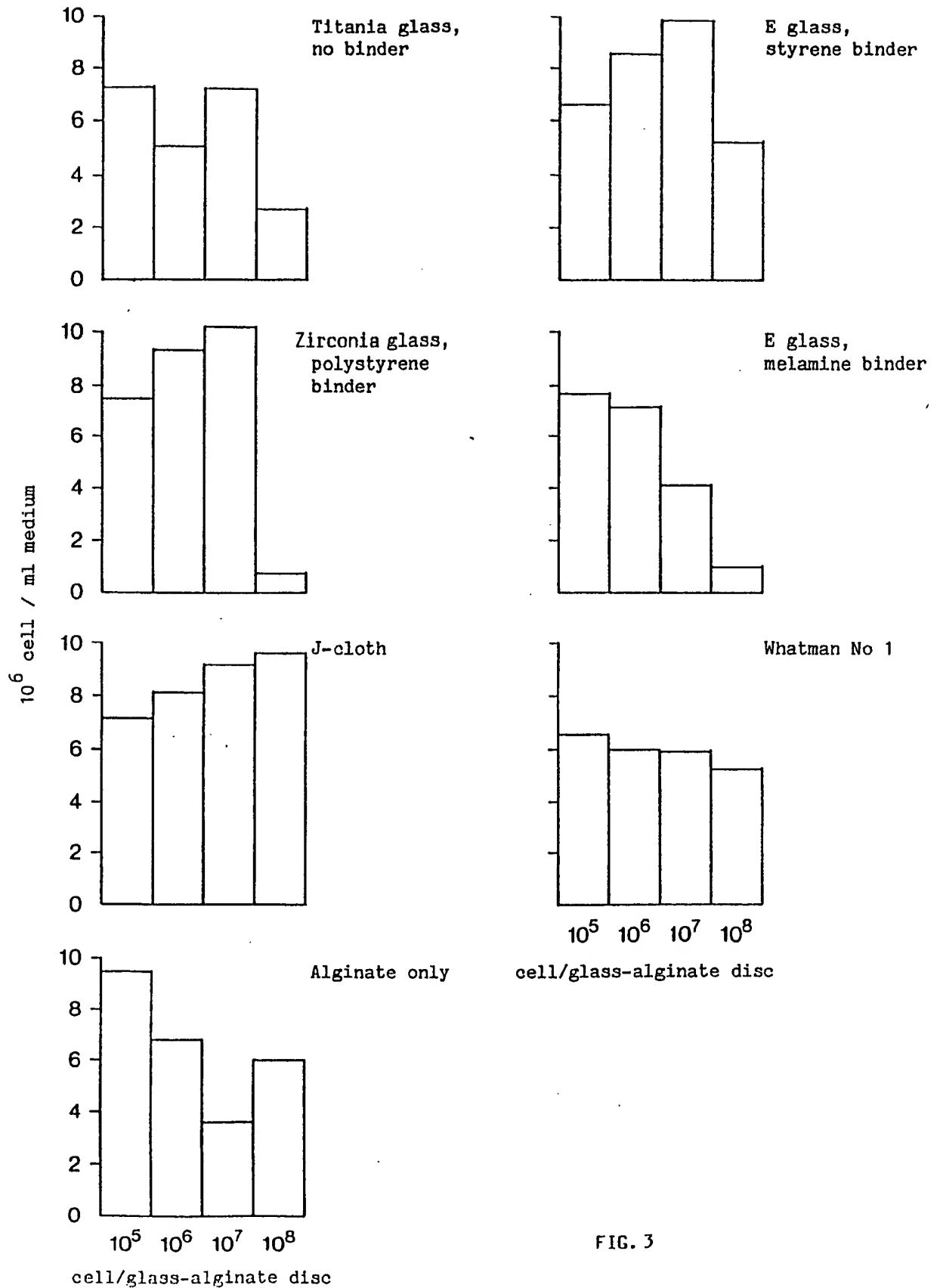


FIG. 3

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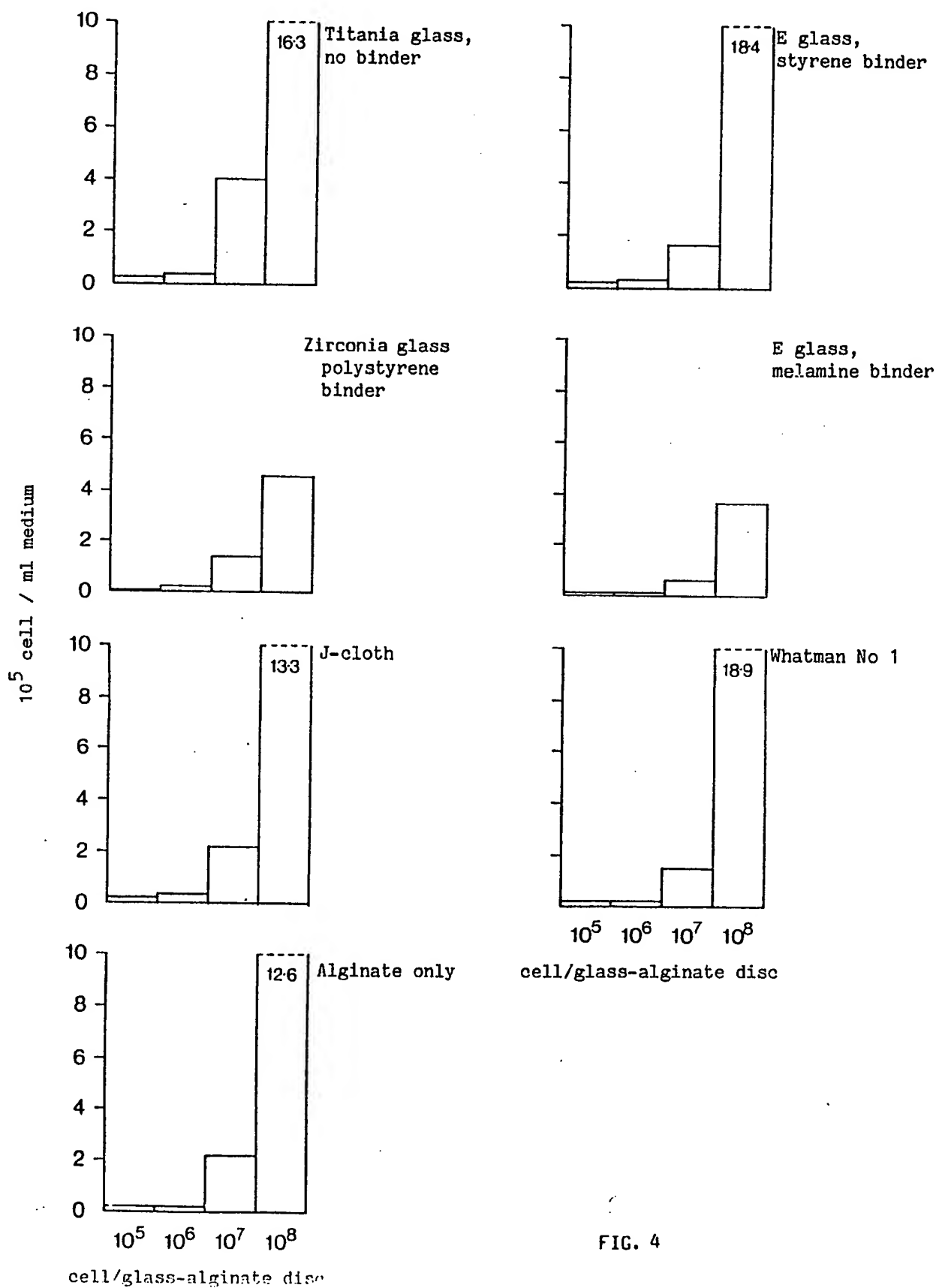


FIG. 4

SPECIFICATION

Improvements in or relating to the growth of plant tissue cultures

- 5 The present invention relates to supporting substrates suitable for use in growing higher plant and algal cells. Such substrates are useful for the production of plant cell products, in particular plant metabolites. Higher plant cells which are isolated from a parent plant can be induced to grow and multiply in or on a suitable substrate to form a growth of plant callus tissue. A callus is an aggregate of substantially undifferentiated, unspecialised plant cells ("callus cells") which are capable, depending upon the physical or chemical environment in which they are placed, of either multiplying or of developing into any of the more specialised plant cells, and, ultimately into a complete plant.
- 10 For many years attempt have been made to recover secondary metabolites from plant tissue cultures. Plant secondary metabolites include a very wide range of galenicals, essential oils and resins which are of great commercial importance in the pharmaceutical, perfumery and flavouring industries. However, attempts to extract such metabolites from suspensions of plant cells have hitherto been limited on a commercial scale, except in the case of one or two types of plant.
- 15 It is generally recognised that plant or algal cells more readily produce secondary metabolites when primary metabolism (i.e. growth) of the cells is inhibited. Thus, if one aims to obtain a high yield of secondary metabolites from immobilised plant cells, it is desirable to use a substrate in which growth of the cells is controlled or inhibited. In order to maintain such high yields over long periods, it is also desirable that leakage of cells from the substrate should be substantially prevented.
- 20 European Patent Specification 52001 describes a system for the growth of plant tissue cultures as calli supported on a substrate in contact with a nutrient medium. The substrates described are either particulate or fibrous, and are rendered capable of carrying the culture by, for example, dipping the substrates in an aqueous solution of a water soluble alginate such as sodium alginate, and then contacting the substrate with a solution of a water soluble non-phytotoxic salt which precipitates an insoluble alginate on the substrate, for instance a water soluble salt of calcium e.g. calcium chloride. The substrate can be inoculated with a culture by suspending the cells in the water soluble alginate solution in which the substrate is dipped.
- 25 There is a general recommendation in this specification to use as the substrate a fibrous material such as glass fibre, cellulose or synthetic organic fibres. However, the manner of use and the form of the glass fibre is not exemplified.
- 30 The substrates which are described in the specific examples of European Patent Specification 52001 are (a) pan scrubbers consisting of an open toroidal bundle of coarse polyamide fibre and (b) fibres of the type sold commercially by Borden under the trade mark "Safe Grow" water mat.
- 35 G.B.2083827B describes the growth of various cell forms, including plant cells, on a two- or three-dimensionally stable material capable of retaining a gel and in fact acting to reinforce the gel, the gel forming the carrier medium for the cells. The material used in the examples is described as a loosely woven cloth, for example that available under the trade name "J-Cloth" from Johnson and Johnson Ltd.
- A widely used method of immobilising algal cells is by forming spherical gel beads incorporating the cells by adding droplets of a solution of sodium alginate containing algal cells to a solution of calcium chloride. This method however suffers from a number of disadvantages, some of which are:
- 40 (a) Cell retention is not effective. Cells are lost from the beads of alginate gel as is evident from the contamination of the incubation medium with free cells which is usually apparent after only one week's incubation.
- 45 (b) The presence of certain materials in the incubation medium can cause the gel to disrupt. Such undesired materials include phosphate, which may be needed where a nutrient incubation medium is used to maintain cell viability.
- (c) The only form of reactor feasible with such beads is a packed bed reactor. This means that problems are experienced in achieving adequate light levels at the centre of the bed, and when photosynthetic gas evolution occurs.
- 50 The above-mentioned G.B. Patent Specification 2083827 suggests the use of a substrate in sheet form which consists of a calcium alginate gel supported on a sheet of cloth sold under the trade name "J-Cloth" by Johnson and Johnson. This proposal however only overcomes the last disadvantage in that it is possible to form an immobilised cell reactor between two glass plates using such sheets. We have found that using an acrylamide polymerised system as described in that specification, additional problems occur due to the high toxicity of the acrylamide monomer.
- 55 We have also found that if the substrate is treated as described at page 3 of European Patent Application 52001, the toxicity problem is avoided but cells are released from the substrate into the incubation medium and there is contamination of the incubation medium with free cells and disruption of the gel in the presence of phosphates.
- 60 We have now found that not only the nature and form of the glass fibre, but the way individual glass fibres have been assembled together to form a compact structure can influence the usefulness of the glass fibre as a substrate for carrying gels in the growth of plant cells.
- There are two main methods of forming glass fibre. One method comprises drawing glass into continuous filaments from a many holed fiberising bushing and sizing the continuous filament material as it leaves the
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- bushing and forming it into a series of strands each made up from a number of individual filaments. Several strands are gathered together to form a roving and wound into a cake. The roving can be chopped up to form chopped strands which can be used as a reinforcing material, or bound together to form a mat known as chopped strand mat. The other method comprises forming separate relatively short lengths of single filament material by using centrifugal force to extrude glass in thin streams from holes in the wall of a rotating spinner, and attenuating these streams using steam or air blasts directed at the streams as they leave the spinner. Another way of forming glass fibre into a close textured matted material is by sizing the filaments with a size which is soluble in water so that chopped strands when placed in water split up into individual filaments and produce a uniform dispersion of glass fibre filaments. This dispersion can then be formed into material known as tissue by forming it into a mat on a porous web in a manner similar to paper making. This mat can then be sprayed with a binder which is cured to cause the filaments to adhere to one another and give a relatively thin but non-paper like material known as glass fibre tissue. This tissue is used primarily in the reinforcement of roofing felt and in the manufacture of lead-acid cells where it is used to maintain the separation between the plates.
- According to one aspect of the present invention, there is provided a process for the production of a composite substrate containing immobilised plant cells, which process comprises contacting a glass fibre tissue (as hereinafter defined) with an aqueous solution of sodium or potassium alginate containing suspended plant or algal cells, and forming an alginate gel on the tissue by contacting the tissue with an aqueous solution of calcium chloride or other suitable non-phytotoxic salt.
- The glass fibre tissue used in the process of the present invention is a glass fibre tissue which has been formed from a dispersion of chopped strands in water, and must be one which is either formed from any available dispersible chopped strand and then treated with a binder which may be a melamine or styrene binder, or if not treated with a binder is formed from glass fibres made by a continuous filament drawing process and formed from a composition containing a transition metal oxide such as ZrO_2 or TiO_2 .
- The composite substrates of the present invention have all of the following advantageous characteristics:
- Low cost
 - Ease of manipulation
 - Low toxicity to cells
 - Exhibit optimum cell retention
 - Possess a form suitable for use in a reactor, and allow adequate light penetration if necessary.
- We have found algal cells (*Chlorella* Spp) to be a satisfactory source of cells for use in the screening of potential composite substrates. Such algal cells exhibit most of the general characteristics of plant cells, in particular with regard to the structure of the cell wall which is believed may be an important factor in the attachment process of a cell to the support matrix. These algal cells are easy to grow in suspension culture, are relatively hardy organisms, and their viability can be easily assessed by available methods. Such methods include oxygen consumption rates, and the production of glycolates, other organic acids or secondary metabolites. Their growth can also be caused to take place in the absence of light in the presence of a suitable carbon source. They are of course also of interest in their own right because of their value as a source of a wide range of secondary metabolites such as glycolic acid, and materials with biocidal activity.
- We have found that glass supports in the form of tissue, chopped strands (before forming into a mat), white wool (i.e. glass wool as used for insulation purposes free of any phenolic binder), porous glass (glass particles which have been phase-separated and then leached to remove the soluble phase and leave an interconnected matrix of holes), and sintered glass beads (small glass beads sintered together to give a porous structure) all have the ability to physically absorb algal cells. However once the cells are so fixed it has been expected that unless cellular reproduction is substantially inhibited, free cells rapidly appear in the incubation medium, thereby indicating that the cells are not retained on the support. The total physical absorptive capacity of any form of untreated glass was found to be low, particularly in the case of porous glass, and in practice it was found difficult to control conditions so that reproduction of the cells was inhibited or controlled without killing the cells or reducing to an unacceptable extent the production of secondary metabolites. In our estimation, there is therefore no advantage to be gained by using the above physical forms of glass as a simple support matrix.
- We have found that certain selected forms of water-laid glass fibre tissues made from dispersed chopped strands used as supports for immobilised cells in an alginate gel give a composite substrate whose performance is unexpectedly superior to that of a composite substrate consisting of plant cells immobilised in alginate gel supported on "J-Cloths" or filter paper, while other forms of water-laid glass fibre tissue formed from dispersed chopped strands treated with alginate perform no better than the said substrates formed from "J-Cloths" and filter paper. In the case of materials which behave in a superior manner there appears to be some form of interaction between the glass, the binder and size materials carried on the glass surface, plant cells, and alginate which enhance the performance of the system.

Most continuous glass filaments are drawn from a composition known as "E" glass. A typical "E" glass composition is:

	SiO ₂	53.4	
5	Al ₂ O ₃	14.4	5
	B ₂ O ₃	8.4	
	MgO	3.1	
	CaO	18.9	
	Na ₂ O	0.2	
10	K ₂ O	0.9	10
	Fe ₂ O ₃	0.4	
	F ₂	0.5	

We have found that a tissue made from "E" glass with the binder removed by burning performs no better than "J" cloth when used as a support for an alginate gel. We have also found that coating the glass fibre with TiO₂ after burning off the binder does not improve the performance of the substrate. We have also found that the performance of a tissue made from "E" glass and coated with an acrylic binder is unsatisfactory. The examples below illustrate the improvement produced by utilising the forms of glass fibre tissue defined above as a substrate in conjunction with an alginate gel for the immobilisation of algal cells.

According to a further aspect of the present invention, there is provided a method for the production of plant cell products, typically secondary metabolites, which comprises contacting a composite substrate produced in accordance with the invention with a liquid nutrient medium, and recovering the desired cell products from the medium.

Plant cells such as the cells of *Capsicum* spp, *Crocus sativus*, *Datura*, *Nicotiana*, and *Triticum* spp can be supported on a glass tissue/alginate gel composite substrate in accordance with the invention for at least three months with satisfactory production of secondary metabolites and with substantially no loss of cells from the substrate (as is evident by the fact that there is no contamination of the circulating medium by live or dead cells). This makes recovery of any secondary metabolites excreted into the circulating medium a relatively more simple procedure than recovery from a medium in which there is contamination with live or dead cells. The retention of the plant cells on the substrate is also of importance in achieving a satisfactory production rate.

Reactors incorporating substrates in accordance with the invention can be formed simply in a flat bed by placing the impregnated glass fibre tissue in a compartment formed between two glass plates which is sealed around the edges except where means are provided for feeding and removing a circulating medium to and from the compartment.

Algae can be grown satisfactorily in vertical reactors. Sufficient space is preferably left to permit circulation of a medium on both vertical faces of the tissue/gel composite so that there is interchange on both faces with the cell material within the alginate gel. The circulating medium can leave nutrients added to it, and materials which cause a change in the nature of the metabolites produced by the algal cells. Algae can be used to produce glycolic acid and materials with an influence on the development of other cell systems which may act as biocides or enzyme inhibitors.

The value of the forms of the fibre glass tissue which are used in accordance with the present invention is primarily in the ability of the glass fibre tissue in combination with an alginate gel to limit but not totally inhibit growth without destroying cells, while at the same time avoiding substantial cell leakage from the gel matrix, even in the presence of phosphates which normally cause the gel to break down.

Material released from the glass or the surface charge pattern of the glass may have an effect in slowing growth without preventing it altogether.

The present invention is illustrated in more detail by the following Experiments.

50 Experiment 1

It was found by monitoring free cell growth in the presence of different forms of glass tissue and in the presence and absence of glucose as a growth promoter, that none of the glass tissues used killed the cells. Thus, "E" glass tissue with the binder removed had little inhibiting effect, and cell growth was close to the normal growth rate in the absence of glucose. "E" glass tissue with a melamine binder inhibited cell growth whether glucose was present or not. This inhibition also occurred if the medium in which the cells were grown was one in which the glass tissue was sealed for four days and then removed. However when the growth of cells and cell leakage was monitored in a comparative test where the substrates were treated with an alginate gel containing the cells, it was found surprisingly that in the case of the "E" glass tissue with a melamine binder, growth took place with substantially no cell leakage. The number of cells appearing in the medium over a period of from 4 to 22 days was monitored and the results are shown in graphical form in Figure 1a. Figure 1b gives the key to the glass tissues used to obtain the results illustrated in Figure 1a. The significant results in the graph are those obtained at 16 days, i.e. at a time before there is evidence of cell death - possibly owing to nutrient limitation - as manifest by the apparent drop in cell numbers at 22 days. The results at 16 days show clearly the improved performance of the substrates in accordance with the invention in contrast to the supports made from filter paper and "J" cloths which were used as controls.

We surmise that the alginate gel may act as a diffusion barrier round the cells thereby permitting material released from the environment of the glass to diffuse only slowly into contact with the cells and thus maintain a low concentration of growth inhibitor over a lengthy period. The alginate gels are conveniently made by any of the known procedures described in the literature. The impregnation of the glass tissue is simply carried out by dipping into a solution of calcium chloride sheets of tissue impregnated with sodium alginate containing the cells which are to be immobilised in the gel.

Experiment 2

Comparative tests have been carried out in which the leakage of cells after ten days from glass tissue alginate matrices into a culture medium containing various levels of phosphate has been compared with leakage of cells from unsupported alginate gels, and from alginate gels supported on "J" cloths, and filter paper.

Phosphate is known to have a disruptive effect of the gel matrix. Phosphate is however required in some circumstances to ensure survival of the cells. The loss of cells in the presence of various concentrations of phosphate from composite substrates of glass fibre tissue/alginate gel has been compared with various control substrates: "J" cloths/alginate gel (control 1), No. 1 Whatman Filter Paper alginate gel (control 2), and alginate gel alone (control 3). The leakage of cells from the substrates after ten days was measured with starting cell concentrations of 10^5 , 10^6 , 10^7 , 10^8 cells per cm^2 of alginate gel. The concentrations of phosphate were $1\mu\text{M}$ and 1mM and 1M . Substrates using four different types of glass fibre tissue were tested. The glass fibre tissues were the following:

- (a) Titania-containing glass with no binder.
- (b) "E" glass with styrene binder.
- (c) Zirconia-containing glass with polystyrene binder.
- (d) "E" glass with a melamine binder.

The cell leakage after ten days from the above and the controls is illustrated by the histograms shown in Figures 2 to 4 of the accompanying drawings. The histograms in Figure 2 show the results obtained with phosphate concentrations of $1\mu\text{M}$; the histograms in Figure 3 show the results obtained with phosphate concentrations of 1mM ; and the histograms of Figure 4 show the results obtained with phosphate concentrations of 1M . The non glass containing supports used as controls showed cell leakage at all phosphate concentrations and at all cell concentrations. It will be seen that at the highest cell concentration which is when cell leakage becomes most obvious all the forms of glass fibre tissue support in accordance with the invention (Samples (a), (b), (c) and (d)) perform much better than the controls, and that support (a) has the best overall performance.

Where callus is grown on glass fibre tissue, the reactor is preferably substantially horizontal, since some forms of callus may not adhere satisfactorily to the bed, and cannot easily be supported without slipping or rolling, if the bed tissue is at too steep an angle (e.g. more than about 30°). However, there is sometimes an advantage in inclining the bed at a very small angle to the horizontal, e.g. 2 to 10° to provide drainage and prevent flooding of the tissue bed from an inequalities of flow.

While there is no theoretical limit of the size of the reactor it will generally be convenient for large scale operation to use relatively small units, arranged in parallel and/or in series. Such arrangements provide for ease of sterilisation and help to localise and isolate any infection. For example, a number of reactors may be supplied from a single reservoir via an inlet manifold and the fluid in the bed may drain, overflow or be pumped into an outlet manifold. Alternatively, it is possible to arrange a number of reactors in cascade, one above the other, allowing the nutrient solution to trickle down the system by gravity. For example, the reactors may be slightly tilted in alternate directions down the cascade so that liquid drains from the lower edge of each reactor into the upper edge of the next lower reactor.

The rate of flow of the nutrient solution through the system should not be so rapid as to disturb or dislodge the culture, but should be sufficient to maintain a substantially constant supply of nutrients to the callus, and to prevent any harmful build up of metabolites. The depth of the nutrient solution should be sufficient to contact and wet the callus tissue without totally immersing it, in order to permit sufficient contact between the cells and the air.

An important advantage of the substrates of the present invention is that they permit the composition of the nutrient regime which is supplied to the culture to be varied in a controlled manner, as desired.

The culture can be fed a solution adapted to promote optimum growth of the unspecialised cells, until a healthy growth has been established, and the composition of the nutrient solution is then gradually altered to optimise production of the desired secondary metabolite. For example, precursors of the desired metabolite or development modifiers such as auxins, cytokinins and gibberellins may be added to the solution, and/or the concentrations of selected nutrients or development modifiers in the solution may be reduced to inhibit competing biochemical pathways.

The invention is particularly useful for systems in which the desired metabolite is excreted into the nutrient medium, and we prefer in such instances to recover the metabolite continually or intermittently from the nutrient medium while it is recycling. This reduces the inhibition of the desired metabolic pathways by feedback mechanisms caused by excessive build up of the metabolite in the nutrient.

Suitable nutrient solutions for the growth of callus cultures are well known in the art. A typical example is described by Marashige and Skoog in *Physiologia Plantarum*, Vol. 15, pp 473-497. Such solutions may be

supplemented by additions of natural plant fluids such as coconut milk, or by yeast or malt extracts.

The supply of air may be controlled in various ways. In general callus culture cannot be conducted in totally anaerobic conditions. Often, sufficient aeration is achieved by permitting some contact between the callus and the atmosphere, or by bubbling air through the nutrient solution or even allowing the nutrient to absorb some air from the atmosphere prior to contacting the calli. Production of secondary metabolites may be favoured by restricting the air supply to the culture.

Illumination can profoundly affect the development of plant tissue cultures. It is sometimes preferred that the vessel containing the culture should be transparent to permit illumination of the growing cells. Normally cultures will be grown in artificially simulated white light, but illumination by selected wavelengths or growth in darkness or under cycles of illumination may in some instances be preferred in order to favour production of specific metabolites.

Preferably, means are provided to control the temperature of the culture, which also affects the rate of growth and the yield of secondary metabolites.

We have found that our invention is useful for the production of a number of plant secondary metabolites. Cells of certain *Capsicum* species e.g. *Capsicum frutescens* or *Capsicum annuum* could be grown on a commercial scale according to our invention and induced to form significant yield of capsaicinoids, the principle active compounds in the flavour of chilli pepper. The term "Capsaicinoids" is used herein generally to include the various components of commercial capsaicin including the compound capsaicin itself, dihydrocapsaicin, nor-dihydrocapsaicin, homo-capsaicin, homo-dihydrocapsaicin.

The production of capsaicinoids is stimulated by the addition of precursors such as valine and phenylalanine to the nutrient solution, once a healthy growth of plant cells has been established. Competing protein synthesis is checked by progressively reducing the nitrate and/or carbohydrate content of the nutrient solution. This may be achieved by allowing the culture to deplete the solution naturally, and then adding the precursor to the nutrient medium. Water soluble salts such as sodium or potassium iso-caprate may also be used as the precursors for the required capsaicinoids.

The substrates of the invention may also be used to stimulate the production of diosgenin by tissue cultures obtained from diosgenin-forming strains of yam of the genus *Dioscorea*. Diosgenin is widely used industrially for the synthesis of certain commercially valuable steroids.

Other secondary metabolites which may be prepared in accordance with the invention include L - DOPA, various alkaloids such as opium alkaloids and derivatives of *Datura*.

Metabolites, typically secondary metabolites, may be recovered from the circulating solution of cultures grown on substrates in accordance with the invention. In many cases the metabolites may be removed from the callus by plasmolysis, which involves contacting the cell with an aqueous solution of a non-toxic, non-ionic compound at a concentration sufficient to promote osmotic shrinkage of the cell, which may then be accompanied by excretion of secondary metabolites.

The following Examples further illustrate the present invention.

Example I

Cells of *Chlorella* spp were dispersed in 33.c.c. of sodium alginate (5% w/v) so as to give a cell concentration of 1.8×10^7 cells/c.c. A rectangular shaped piece of glass fibre tissue (14cm x 13cm) made from E glass with a styrene binder and sold under the trade description E100M by Regina Fibreglass Ltd. was loaded with the dispersion by coating the dispersion onto the piece of glass fibre tissue. The coated glass fibre tissue was then dipped in a solution of calcium chloride. It was left in the calcium chloride solution until a satisfactory gel had formed and then removed and placed in a compartment formed from two glass plates spaced about 10 mm apart and sealed around their peripheral outer edges except where means were provided to pass a circulating medium in and out of the space between the plates. A *Chlorella* growth medium was circulated through the compartment in contact with the gel for 21 days at a temperature of 25°C. The compartment was illuminated with a fluorescent lamp.

There was substantially no leakage of cells into the medium over a period of 21 days.

Example II

The non-toxic nature of the glass fibre tissue/alginate gel matrix used in Example I to plant cells was demonstrated by showing that calluses of the plants *Datura*, *Nicotiana*, and *Triticum* survived for three weeks while supported on a small piece of the reinforced gel matrix in a flask along with an appropriate liquid medium. The flask was kept agitated at a temperature of 25°C for the whole three weeks. No signs of cell leakage were found with any of the plant cells tested.

CLAIMS

1. A process for the production of a composite substrate containing immobilised plant cells, which process comprises contacting a glass fibre tissue (as hereinbefore defined) with an aqueous solution of sodium or potassium alginate containing suspended plant or algal cells, and forming an alginate gel on the tissue by contacting the tissue with an aqueous solution of calcium chloride or other suitable non-phytotoxic salt.
2. A process according to Claim 1, wherein the glass fibres are made from an "E" glass composition.

3. A process according to Claim 2, wherein the composition of the "E" glass is:

	SiO ₂	53.4	
	Al ₂ O ₃	14.4	
5	B ₂ O ₃	8.4	5
	MgO	3.1	
	CaO	18.9	
	Na ₂ O	0.2	
	K ₂ O	0.9	
10	Fe ₂ O ₃	0.4	10
	F ₂	0.5	

4. A process according to any one of the preceding claims, wherein the plant or algal cells are cells of the species *Chlorella*, *Capsicum*, *Crocus*, *Datura*, *Nicotiana*, *Triticum*, and *Dioscorea*.

15 5. A process according to Claim 1 substantially as hereinbefore described with reference to Experiment 1 or 2 or Example 1 or 2. 15

6. A composite substrate of a glass fibre matrix carrying plant or algal cells immobilised thereon whenever prepared by a process as claimed in any one of the preceding claims.

7. A method for the production of plant or algal cell products, which comprises contacting a composite substrate as claimed in claim 5 with a liquid nutrient medium, and recovering the desired cell products from the medium. 20

8. A method according to Claim 7, wherein the cell products are secondary metabolites.

9. A method according to Claim 8, wherein the cell products are capsaicinoids, diosgenin, L-Dopa, opium alkaloids, or derivatives of *Datura*.

25 10. A method for the production of plant or algal cell products substantially as hereinbefore described with reference to Example 1. 25

11. Plant or algal cell products, whenever obtained by a method as claimed in any one of claims 7 to 10.